EXTENDED EXPERIMENTAL PROCEFURES

Mice and AAVs

All experimental procedures were performed in compliance with animal protocols approved by the IACUC at Boston Children's Hospital. BCL-2^{+}/$PTEN^{f/f}$/$SOCS3^{f/f}$ mice were first obtained by crossing male BCL-2 over-expressing mice, in which transgene expression is controlled by the neuron-specific enolase promoter (Chen et al., 1997), with female $PTEN^{f/f}$/$SOCS3^{f/f}$ mice (Sun et al. 2011), and maintained by crossing BCL-2^{+}/$PTEN^{f/f}$/$SOCS3^{f/f}$ males with $PTEN^{f/f}$/$SOCS3^{f/f}$ females. Production of AAV2/2-CAG-Cre, PLAP, CNTF, OPN (Duan et al. 2015), IGF1 and ChR2-mCherry (titer of each: 10^{12}–10^{13} genome copies per ml) was performed by the Viral Core at Boston Children's Hospital. The vector of AAV2-CAG-ChR2-mCherry was constructed by linking the CAG promoter with a sequence of humanized channelrhodopsin-2 with H134R mutation fused to mCherry that was originally from the laboratory of Karl Deisseroth (Addgene 20938).

Young SC injury model

A BCL-2^{+}/$PTEN^{f/f}$/$SOCS3^{f/f}$ pup at postnatal day 6 was anesthetized using hypothermia, and a bone flap was made immediately caudal to the transverse dural sinus visible through the skull, to reveal the underlying right SC including its lateral and medial borders. After lifting of the right transverse dural sinus with fine forceps, a curved knife made from stainless steel razor blades (Ted Pella Inc.) was inserted underneath the elevated sinus and the cortex. With the tip of the knife 1-1.5 mm deep into the SC, a cut was made across the rostral end of the SC. The cut typically started from a point lateral to the SC and then proceeded towards and beyond the medial border of the SC, in order to completely transect the retinocollicular axons in the right SC. In control mice, such procedure typically induces extensive axon retraction from

the lesion site; denervation of visual inputs may also take place for visual targets in the vicinity of the SC such as the pretectal nuclei. Bleeding was quickly stabilized by applying Gelfoam (Pfizer Inc.) pre-planted near the edge of the cranial window. The bone flap was then replaced and the skin closed with an 8.0 suture (Ethicon Inc.). Local analgesic was applied around the sutured area. The pup was then warmed up on a warm blanket before being returned to its mother. 30-60 min after SC injury, the operated pups were re-anesthetized, and 1-1.5 µl AAV-Cre (i.e. regeneration group), or AAV-PLAP (i.e. control group) was injected intravitreously into the left eye, after the closed eye was surgically opened. The efficiency of such AAV-Cre application in deleting a floxed gene was examined in pilot experiments using the Rosa-LSL-tdTomato reporter line (Jackson Laboratory): over 80% of the total retinal ganglion cells were estimated to express Cre two weeks after injection. In some experiments, 0.5-1 µl cholera toxin beta subunit (CTB-488, 1 µg/µl, Invitrogen), was injected with a similar method for tracing the retinocollicular axons. One week after SC injury, the operated pups were anesthetized again with ketamine (100 mg/kg) / xylazine (10 mg/kg). For mice in the regeneration group, 1 µl AAV-CNTF was injected intravitreously into the left eye to boost axon regeneration. When ChR2-based electrophysiology was to be performed, 1 µl AAV-ChR2-mCherry was injected intravitreously into the left eye, either in a mixture with 1 µl AAV-CNTF for mice in the regeneration group, or in a mixture with 1 µl AAV-PLAP for mice in the control group. Care was taken not to damage the eyeballs during injections. The retinocollicular axons were visualized by anti-mCherry immunostaining in AAV-ChR2-mCherry-injected mice, or by intravitreous injection of 1 µl CTB in adult mice two days prior to tissue harvest.

Adult SC injury model

Adult PTEN$^{f/f}$/SOCS3$^{f/f}$ mice (8 weeks after birth) were anesthetized with ketamine (100 mg/kg) / xylazine (10 mg/kg). Intravitreous injection into the left eye was performed with

either 1 μl AAV-Cre mixed with 1 μl AAV-CNTF (i.e. regeneration group), or with 2 μl AAV-PLAP (i.e. control group), either two weeks before optic tract crush (pre-treatment) or two days after crush (post-treatment). A cranial window was created over the right cortex by removing a bone flap and cortical/hippocampal tissues were aspirated to reveal the right optic tract entering the SC. The exposed optic tract was then completely crushed by a pair of modified forceps (Dumont #5). Breeding was stabilized and blood clot was cleared away. The surgical cavity was then filled with clean Gelfoam, the bone flap put back, and the skin sutured. Dexamethasone (2.5 mg/kg) was administered subcutaneously to reduce brain edema. Standard post-op care was then followed.

About two weeks after injury, 2 μl AAV-ChR2-mCherry was injected intravitreously into the left eyes of all mice under general anesthesia. The retinocollicular axons were visualized by the antibody-enhanced mCherry tag in AAV-ChR2-mCherry, or by intravitreous injection of 1 μl CTB two days prior to tissue harvest during model characterization.

In the AAVs-OPN/IGF1/CNTF experiment, adult wild-type mice were subject to optic tract transection as described. The injury site was placed adjacent to the lateral geniculate nucleus (i.e. more proximal to the SC) to increase the length that regenerated retinal axons need to travel before interacting with the SC as well as the pretectal nuclei. Such length was estimated to be about 2 mm for the SC. Two days after optic tract transection, a mixture of AAV-OPN, AAV-IGF1 and AAV-CNTF (1 μl each; AAV-PLAP as control) was injected intravitreously into the contralateral eye, followed by AAV-ChR2-mCherry injection another two weeks later. Mice were then subject to tests 15-16 weeks after injury.

Demyelination model

Two weeks after an adult wild-type mouse received intravitreous injection of AAV-ChR2 in the left eye, the right optic tract proximal to the SC was exposed as described above. Micro-injection of 1% Lysolecithin (also called lysophosphatidylcholine or LPC, Sigma; in 0.9% saline) was performed using Nanoliter Injector (WPI) across the whole tract (0.5 μl x 3) close to the dLGN. Behavioral, electrophysiological and histological analyses were performed 1-3 weeks after injection.

Optic nerve crush model

Adult PTEN$^{f/f}$/SOCS3$^{f/f}$ mice were anesthetized with ketamine (100 mg/kg) / xylazine (10 mg/kg). Using two pairs of Dumont #5 forceps (FST), the optic nerve was exposed intraorbitally through a surgical opening immediately behind and above the eyeball, and crushed approximately 0.5-1 mm behind the optic disc. AAVs-OPN/IGF1/CNTF, AAVs-Cre/CNTF or AAV-PLAP was injected intravitreously 2 weeks prior to optic nerve crush.

12 days after crush, intravitreous injection of CTB-555 (1 μg/μl, Invitrogen) was performed to trace axons in the optic nerve. Two days later, mice were perfused with 4% paraformaldehyde and the optic nerves dissected out and cryosectioned into 10 μm longitudinal sections. The sections were then washed with PBS, mounted and imaged with confocal microscopy. For estimation of the number of regenerating axons at each distance from the crush site, the cross-sectional width (*width*) of the nerve was measured and the number (*n*) of CTB labeled axons at such distance was counted. Given the thickness of the section (*thickness*, or 10 μm), the "density" of axons at such distance was calculated as *n*/(*width* x *thickness*). Assuming a cylindrical structure of the nerve, the total number (*N*) of axons at such distance was calculated as

$$N = \frac{n}{width \times thickness} \times \pi \times (\frac{width}{2})^2 = \frac{\pi \times n \times width}{4 \times thickness}$$

Tissue harvest, staining and imaging

Mice were transcardially perfused with 4% paraformaldehyde, and tissues were harvested and post-fixed overnight at 4 °C. For vibratome sectioning (Leica), the brain tissue was pre-embedded in 1.5% agarose, and serial parasagittal sections (200-250 μm) were collected and imaged using confocal microscopy. For cryosectioning, tissues were kept in 30% sucrose for 48 hours before they were embedded in OCT compound and snap frozen with dry ice. For the SC, serial sagittal sections (25 μm) were collected. Primary antibodies used for staining includes: goat CTB (1:4000; List Biological Laboratories), rabbit GFAP (1:500; Dako), rat GFAP (1:500; Invitrogen), rabbit red fluorescent protein (mCherry, 1:400; Abcam), mouse NeuN (1:200; Millipore), rabbit P-S6 (Ser235/236) (1:200, Cell Signaling), mouse neuronal class β–III tubulin (Tuj-1, 1:400; Covance), mouse MAG (1:100, Millipore), rat MBP (1:100, Millipore). For SC staining, standard immunostaining protocol was followed. Briefly, sections were incubated for 1-2 days at 4 °C with primary antibodies, washed three times for 20 min with PBS, and then incubated with secondary antibodies (1:200, Jackson ImmunoResearch) for 1 h at room temperature. Some sections were then counter-stained with fluorescent Nissl (Invitrogen) after further washing. TSA system (Perkin Elmer) was used to amplify the CTB signal according to the manufacturer's protocol. For retinal staining, eyes were either cryosectioned (16 μm) and stained for immunohistochemistry, or the dissected retina was stained as a whole.

Fluorescent images were acquired using Zeiss fluorescent confocal microscopy (LSM 700). Figures showing large brain areas were acquired using Ultraview Vox Spinning Disk Confocal Microscope with automatic image stitching software Volocity (PerkinElmer). All images were analyzed with Image J (NIH).

Quantification of axon regeneration in the SC

The intensity of CTB or ChR2-mCherry-labeled regenerating axons was estimated at different distances (*d*) from the lesion site using ImageJ. For each section, the starting point (*d* = 0 mm) was defined as the point where the lesion scar intersected with the dorsal surface. A straight line ("reference line") was then drawn in rough parallel with the lesion scar at the starting point and total pixel number in the superficial SC was obtained after the subtraction of background. Such pixel number was defined as the intensity of labeling at *d* = 0 mm. The same method was used to calculate the total pixel numbers across the regeneration pathway. For each animal, total pixel numbers for individual distances were added together for every 7^{th} section, and normalized as percentages relative to the value (100%) obtained at the pre-lesion intact site (*d* = -0.02 mm for the model where the lesion was close to the SC and -0.5 mm for the model where the lesion was far from the SC). The percentage values were then averaged over all animals in each group.

LFP recording *in vivo*

A mouse with AAV-ChR2-mCherry was anesthetized initially using Nembutal (50 mg/kg, I.P.), Dexamethasone (2.5 mg/kg, S.C.) and Chlorprothixene (10 mg/kg, I.M.), supplemented with 0.2-0.5% isoflurane (in 100% O_2). The mouse head was then fixed in a stereotaxic frame, while the body maintained at 37^{o}C by a heating pad with constant monitoring of the rectal temperature. Heart rates, blood oxygenation levels and breathing rates were monitored throughout the recording. A cranial window was opened above the SC and a part of the occipital cortex was carefully removed by suction to expose the SC. Tissue discontinuity and scaring aided the identification of the lesion and post-lesion recording was performed at least 300 μm caudal to the lesion to avoid potential "leakage" of stimulating light into the pre-lesion intact area. A glass pipette recording electrode (2-4 MΩ) filled with 1 M NaCl was then inserted

vertically into the exposed SC. To record terminal-evoked LFPs, an optical fiber (0.5 mm diameter) was placed at an angle ~15-20^{o} immediately next to the recording electrode with the tip of the fiber almost touching the SC. A light pulse (5 ms) generated by a 470 nm diode pumped crystal laser (CrystaLaser, NV) was used to illuminate the area with the same constant output. LFPs were band-pass filtered (0.1-100 Hz), amplified and fed to a computer for recording and analysis. At least twenty events were averaged in synchrony with the laser stimulus onset to obtain the LFP waveforms. After the terminal-evoked LFP was recorded, the tip of the optic fiber was then repositioned to a point adjacent to the center of the pupil of the contralateral eye (without moving the recording electrode) to record the "paired" eye-evoked LFP. In some mice, similar terminal-/eye-evoked LFPs were also recorded in the pre-lesion areas rostral to the lesion site. In cases of small LFPs (<20 μV), one hundred to two hundred events were averaged to obtain the final LFP waveforms. Local applications of 4-AP (Sigma), 4-AP-3-Me (Santa Cruz) and kynurenic acid (Sigma) were performed by "bathing" the exposed SC in respective solutions.

The person that performed the recording was blinded to the mouse genotype and treatment. Multiple sites of the exposed SC were "sampled" to record terminal-evoked LFPs. To compare the sizes of the terminal-evoked LFPs among animals, the maximal amplitudes recorded from individual animals were used.

Whole-cell intracellular recording *in vitro*

Deeply anesthetized mice (4-6 weeks old) were transcardially perfused with about 25 ml of an ice-cold and oxygenated (95% O_2, 5% CO_2) cutting solution containing (in mM): 95 N-Methyl-D-glucamine, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 10 $MgSO_4$, 0.5 $CaCl_2$, 25 Glucose, 5 Na-ascorbate, 3 Sodium Pyruvate, 2 Thiourea. The pH of the solution was titrated to 7.3-7.4 with concentrated HCl (about 8 ml of 10 M HCl

per liter) and the osmolarity was adjusted to 310-315 mOsm. The brains were rapidly removed, embedded in 2% low-melt agarose, and 300-350 μm parasagittal slices containing the SC were prepared in the same cutting solution using Compresstome (VF200, Precisionary Instruments). Slices were then incubated in the same cutting solution at 31-32 °C for 15 min, before being transferred into a holding chamber containing room temperature oxygenated recording solution of the following composition (in mM): 125 NaCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 1 MgSO4, 2 $CaCl_2$ and 25 Glucose (pH 7.3-7.4, with osmolarity of 310-315 mOsm). The slices were stored for 1 h prior to use for recording. 3-5 slices containing the SC region were typically produced from each animal, and recording was made on the slices obtained from the middle SC region. 100 μM picrotoxin (Tocris Biosciences, MO) was added to the recording solution to block GABA receptor mediated responses. Whole-cell patch clamp recording of synaptic responses was made using 2-4 mΩ glass pipettes with internal solution of (in mM) 125 Cs.F,10Cs.Cl, 10 EGTA, 10 HEPES (pH 7.3). The lesion site was identified on the basis of tissue discontinuity and scarring, which was confirmed by post-recording GFAP staining. Blue stimulation light was produced by a 470 nm diode pumped crystal laser (CrystaLaser, NV) and applied through an optic fiber (Thorlabs, NJ). Stimulation duration at 2 ms was found to be able to saturate postsynaptic responses recorded, and therefore was chosen. Neurons had input resistances in a range of 1-5 GΩ and series resistances less than 30 MΩ. To avoid potential complications resulting from well-recognized reduction of tissue viability in mature adult slices, series resistance was measured throughout experiments and only cells that remained within these bounds were analyzed. There was a relatively low efficiency in finding “good” cells in the injured mature SC, particularly in the areas close to the lesion. The membrane potential was first held at -70 mV to record the evoked AMPA receptor-mediated synaptic currents (NMDA receptors were presumably blocked by magnesium at this holding potential). The membrane holding potential was

then switched to +55 mV to recording a mixture of AMPA and NMDA receptors-mediated currents. The total number of cells analyzed was counted to generate N. Applications of the AMPA receptor antagonist CNQX (Tocris) and the NMDA receptor antagonist D-APV (Tocris) were performed by adding respective drugs into the bathing recording solution. All recordings were made with an Axopatch700B amplifier and digitized using a Digidata1400 analog-to-digital board. Stimulation and data acquisition were performed with pClamp software and digitized at 10 kHz. All equipment and software are from Axon Instruments/Molecular Devices (Molecular Devices, CA).

Optomotor test

Optomotor test was performed by an independent experimenter in a blinded fashion using a virtual optomotor system (CerebraMechanics Inc.). A virtual cylinder comprising a vertical sine wave grating was projected in two-dimension on four computer monitors arranged around a testing arena located inside a soundproof box. A video camera was positioned directly above the animal. A rotating (12 deg/sec) grating perceptible to the mouse was projected on the cylinder wall, and the mouse was allowed to track the grating with reflexive head movements in concert with the rotation. The experimenter blinded to genotype and drug treatment scored if animals tracked the moving grating. To measuring acuity, a homogeneous gray stimulus was first projected on the cylinder, followed by a low spatial frequency (0.05 cyc/deg) sine wave grating (100% contrast) of the same mean luminance and moving in one direction. The animal was assessed for tracking behavior for 3-5 seconds, and then the gray stimulus was restored. The short testing epochs reduced the possibility of the mouse's adapting to the stimulus. The spatial frequency of the grating was then systematically increased through a method-of-limits procedure until the animal no longer responded. A threshold for each direction of rotation was assessed this way, and the highest spatial frequency tracked in either direction was recorded as the corresponding eye's acuity. The test normally lasted for 5

to 10 minutes per mouse. All tested mice were over two months old. The injured eye means the left eye contralateral to the injured right SC. The intact eye means the un-treated right eye; some of its retinal axons to the right SC were also presumably injured but the acuities were found to be comparable to naïve un-operated mice. Compounds were administered intraperitoneally (I.P.) 3 hours prior to testing.